Soft Matter

Swelling characteristics of DNA polymerization gels†

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The development of biomolecular stimuli-responsive hydrogels is important for biomimetic structures, soft robots, tissue engineering, and drug delivery. DNA polymerization gels are a new class of soft materials composed of polymer gel backbones with DNA duplex crosslinks that can be swollen by sequential strand displacement using hairpin-shaped DNA strands. The extensive swelling can be tuned using physical parameters such as salt concentration and biomolecule design. Previously, DNA polymerization gels have been used to create shape-changing gel automata with a large design space and high programmability. Here we systematically investigate how the swelling response of DNA polymerization gels can be tuned by adjusting the design and concentration of DNA crosslinks in the hydrogels or DNA hairpin triggers, and the ionic strength of the solution in which swelling takes place. We also explore the effect hydrogel size and shape have on the swelling response. Tuning these variables can alter the swelling rate and extent across a broad range and provide a quantitative connection between biochemical reactions and macroscopic material behaviour.

Introduction

Hydrogels are critical components of biological implants, drug delivery and tissue constructs, and soft robots.[1–4] Responsive hydrogels can change their volumes significantly in response to environmental alterations, such as pH, light, and temperature, because of the absorption or release of water.[5–7] More recently, hydrogels have been developed that can swell or dissolve in response to biomolecular signals such as enzymes, antibodies, and nucleic acids.[8–11]

Most structures in which responsive hydrogels are integrated require a specific speed and extent of response. Thorough characterization of the temperature-dependent swelling of poly(*N*-isopropylacrylamide) (PNIPAM) enabled the construction of pNIPAM-based robotic actuators and devices for robotics and biomedical applications.[12–15]

Here, we investigate the factors that can be used to tune the extent of polymerization gel expansion in response to DNA sequence triggers. DNA-based hydrogels are especially promising for biomedical applications due to DNA's innate biocompatibility and large and continuing-to-be-developed set of tools/controllers for interfacing with other biomolecules that can direct their shape or size change.[16–18] DNA-crosslinked hydrogels that respond to temperature, ions, and small molecules have been reported. This work focuses on DNA-crosslinked polyacrylamide hydrogels, termed DNA polymerization hydrogels, that can expand up to 100-fold volumetrically because of the sequential incorporation of DNA hairpins into their DNA crosslinks (Fig. 1a).[19–22] A family of these DNA polymerization gels where each gel consists of a DNA duplex crosslinks and a polymer backbone made from mono/macromers such as acrylamide (Am), acrylamide-*co*-bis-acrylamide (Am-BIS), poly(ethylene glycol) diacrylate (PEGDA), and gelatin-methacryloyl (GelMA) have also been developed.[22–24] The resulting multi-component DNA polymerization gels have a broad range of mechanical properties and enhanced biocompatibility. Gel photo-patterning protocols for each hydrogel make it possible to assemble these gels at micron-scale in custom shapes.

To achieve more complex movements of the hydrogel system, we designed a mechanism that allows reversible hydrogel shape

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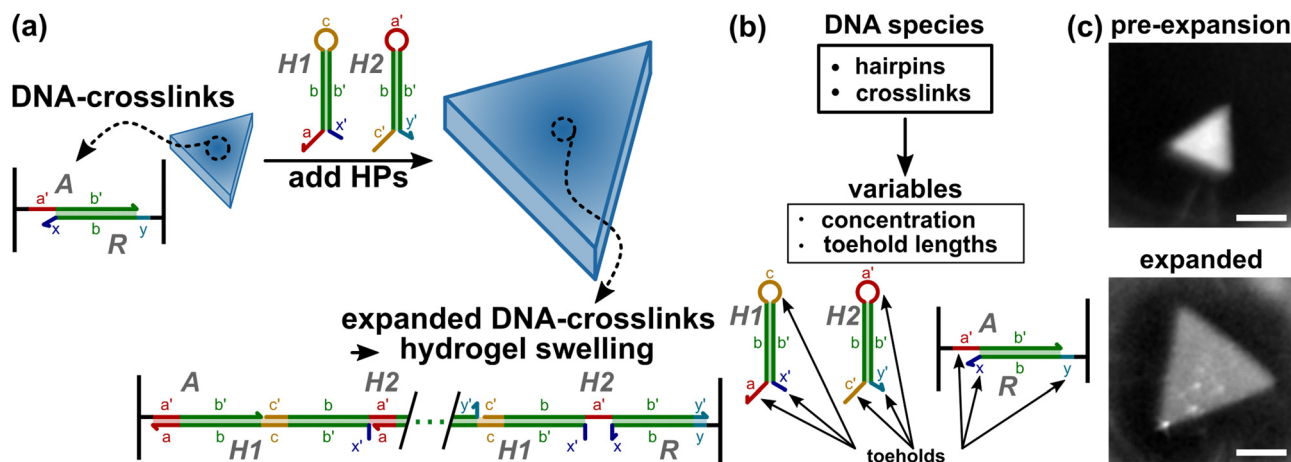
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Fig. 1 Schematic of DNA polymerization gel swelling using DNA hairpins, and tuning swelling by varying the concentrations or sequences of the DNA hairpins or crosslinks. (a) The hydrogels contain anchored DNA crosslinks that initiate the hybridization chain reaction (HCR) in which DNA hairpins polymerize. DNA hairpins sequentially incorporate into crosslinks, thus extending the resulting chain, significantly increasing the DNA content within the hydrogel. (b) Variables that could control the rate and degree of DNA–induced hydrogel swelling. (c) Representative optical images of a poly(PEGDA10k-*co*-S1dsDNA1.154) hydrogel expanded using 20 μM of each hairpin (H1 and H2) before and after polymerization. Hydrogels are visualized using Rhodamine B methacrylate that is incorporated during polymerization (see Materials and methods, ESI†). Scale bars: 1 mm.

change: one set of DNA triggers swelling, whereas another set triggers shrinking.[25] Using these triggers, gels with different polymer backbones can be reversibly actuated for multiple cycles. Finally, we have achieved the design and fabrication of microscale devices, *i.e.*, gel automata, that can utilize chemical systems for swelling and shrinking to control multi-directional, multi-step motion in cycles and transform into meaningful shapes such as letters of the alphabet or numbers.[25]

The swelling process of DNA polymerization gels is triggered by DNA hairpins approximately 50–70 bases in length (Fig. 1a). DNA hairpins insert into the hydrogel *via* a strand displacement reaction between a DNA hairpin and the DNA crosslink or growing DNA chain.[22,26] The thermodynamics and kinetics of strand displacement reactions are well-studied, and the dependence of these parameters on the lengths and sequences of the different domains of the hairpin and crosslink sequences have been elucidated.[27–29] For example, the lengths of the toeholds can determine the rate of the insertion reaction across multiple orders of magnitude and can be tuned independently of the thermodynamics (*i.e.*, the energy change) of the reaction.[27,28] The ionic strength has also been shown to affect the rate of DNA strand displacement.[28,30]

Here, DNA strand displacement occurs inside a hydrogel network, and its procession is coupled to a change in network size and density. We sought to understand how the thermodynamics and kinetics of the chemical reaction that governs insertion affect the rate and extent of the hydrogel swelling process. To do so, we systematically investigated how different features of the DNA polymerization process affect how quickly and how much swelling of hydrogels occurs. We study the DNA-directed swelling of photopatterned poly(ethylene glycol)-*co*-DNA (PEG-*co*-DNA) hydrogels.[23] These hydrogels have both covalent crosslinks between PEG chains that are not stimulus-responsive and DNA crosslinks that respond to specific DNA signals. We measure variations in the swelling rate and extent of these PEG-*co*-DNA hydrogels that result from changes in the thermodynamics and kinetics of the DNA strand displacement reactions that drive swelling.

We investigate how the concentrations of the DNA cross-links and DNA hairpins and domain lengths on the hairpins that initiate strand displacement affect swelling. Well-mixed solution kinetics would predict that the flux of polymerization, and thus (assuming it is reaction-limited) the rate of swelling, should be proportional to the product of the crosslink and hairpin concentrations.[31] We also consider the effect of toehold sequence on the swelling process. Toehold sequence and length are known to be primary determinants of the kinetics of both 3-way and 4-way toehold-mediated branch migration processes, where in 3-way branch migration, the log of the forward rate of interaction is proportional to toehold length.[27,28,32] We then consider the influence of salt concentration since the kinetics and thermodynamics of the DNA polymerization process that drives swelling could be affected by cation type and concentration (as could the degree of polymer network expansion).[30,33] Given that the PEG gels expand considerably despite the constraints on network topology induced by the PEG–PEG chain crosslinks, we test whether DNA-directed swelling could occur in other means besides the extension of crosslinks. Finally, we explored the impact of morphological attributes on the reaction kinetics as the materials' diffusion time is reliant on the shape and size.[34]

Our results suggest that the amount of energy expended in the polymerization of crosslinks has a direct and proportional effect on the amount of swelling of the hydrogels. The rate of polymerization can also affect the rate of swelling. However, given the interplay of the polymerization reaction and other reactions and forces involving the DNA strands, polymer network, and ions, a quantitative theory for tuning these forces by designing DNA must take all of these factors into account.

Results

PEG-*co*-DNA polymerization hydrogels

In this paper, we utilized PEG-*co*-DNA polymerization hydrogels in our studies. The macromer, polyethylene glycol diacrylate (PEGDA10k, M_n 10 000), was used for building the base polymer matrix. Due to the presence of two acrylate moieties on each end of the PEG chain, PEGDA can form a polymer network through free radical-initiated chain polymerization without the presence of DNA crosslinks.[23,35] This property enables us to vary the amount and form of DNA (*e.g.*, single-stranded or double-stranded) anchored inside the gel without compromising the integrity of the polymer network. We used a double-stranded DNA complex (dsDNA) composed of annealed A and R strands that were both short, synthetic DNA and were purchased from IDT as a DNA hybridization chain reaction (HCR) initiator. [dsDNA] refers to the concentration of the double-stranded DNA complex, which we assume is the same as the concentrations of A and R strands. We first polymerized PEGDA10k hydrogels using a pre-gel solution containing 1.154 mM of the dsDNA into equilateral triangle-shaped gel sheets (1 mm in side length and 160 μm in thickness) using a previously developed hydrogel photopatterning method.[22,23] All studies in this work used this structure to measure swelling rate and extent unless otherwise stated. Briefly, the photopatterning chamber consists of a chromium (Cr) CAD photomask and a glass substrate, with 160 μm-thick spacers that control the height of the gels. During photopatterning, the pre-gel solution, which contains the PEGDA macromers, acrydite-modified DNA crosslinks, photoinitiator, and fluorophore, was exposed to UV light through the transparent parts of the Cr mask and thus cured. Photopatterning the structures used in our study allowed the use of gels of precisely the same sizes for comparison, minimizing one potential source of variability in swelling behavior. We first verified that the poly(PEGDA10k-*co*-S1dsDNA1.154) hydrogels expanded in response to DNA by incubating the hydrogels in a DNA hairpin solution. This solution used for swelling contained 20 μM of each of two DNA hairpins, H1_S1_6/3 and H2_S1_6/3 (sequence set 1, Table S1, ESI†), which was previously shown to incorporate into the DNA crosslinks *via* insertion polymerization.[22,26] The volume of the DNA hairpin solution used for each triangle-shaped gel sheet swelling was 100 μL, while the volume of the as-made gel is about 0.08 μL. There are roughly 30 times more DNA hairpins in the solution as a whole than there are DNA crosslinks, which are only inside the gel (Text S1, ESI†). The gels maintained their triangular shape and almost doubled their side length after 60 hours of hairpin incubation (Fig. 1c). No swelling behaviour was observed when only buffer or the wrong sequences of DNA hairpins were present (Fig. S1a, ESI†).

Variation of swelling when varying DNA crosslink and hairpin concentrations

We used the PEG-*co*-DNA gels to investigate the effect of DNA crosslink concentration on the degree and rate of DNA-induced hydrogel swelling. We prepared poly(PEGDA10k-*co*-S1dsDNA)

hydrogels using pre-gel solutions containing 0.250, 0.750, or 1.154 mM double-stranded DNA crosslinks (A_S1 and R_S1) and incubated each of the hydrogels with 20 μM of each of the set 1 sequence hairpins, H1_S1_6/3 and H2_S1_6/3 (Fig. 2a). We plotted the swelling ratio $\Delta L/L_0$ (the change in the side length with respect to the original side length L_0) as a function of time (hours). The initial rates of swelling, *i.e.*, the average rate of swelling per hour over the first two hours, were 0.020 ± 0.002, 0.064 ± 0.007, and 0.046 ± 0.002 (± represents standard error), respectively, for the three crosslink concentrations (0.250, 0.750 and 1.154 mM dsDNA anchors respectively) calculated using MATLAB's polyfit function. This data suggest that lower crosslink concentrations reduce the rate of swelling but do not fit any definite trend, perhaps because the initiation rate is subject to variation because of mixing time, and relatively few data points are used to estimate the slope. The relative changes in side length after 60 hours of incubation with hairpins, which we chose as an approximation to the final state swelling extent, were 0.23 ± 0.03, 0.65 ± 0.17 and 0.82 ± 0.06 (95% confidence interval) for hydrogels containing 0.250, 0.750 and 1.154 mM dsDNA crosslinks respectively. These extents suggest that the total swelling is roughly linear in the crosslink concentration over the range of concentrations tested. Such a result would be consistent with the idea that DNA hairpins are in excess in these experiments so that each crosslink swells to roughly the same extent regardless of crosslink concentration and each crosslink contributes roughly the same amount on average to the extent of the swelling increase of the hydrogel.

The above argument assumes that DNA hairpins are in excess, so raising the hairpin concentration should not affect the final extent of swelling. A higher hairpin concentration could, however, affect the rate of achieving that extent by speeding up the rate of polymerization at crosslinks. We evaluated these hypotheses by repeating the swelling of PEG-*co*-DNA hydrogels with different concentrations of crosslinks but with 60 μM (rather than 20 μM) of each hairpin type (Fig. 2b). Both the initial
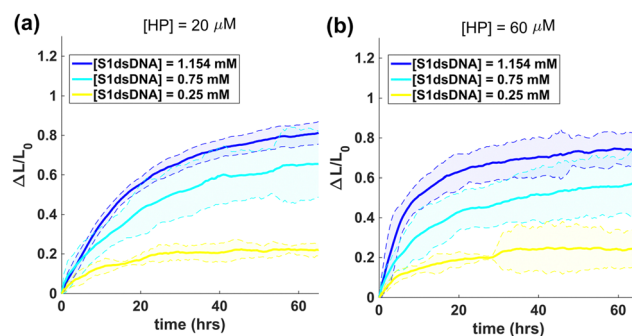
Fig. 2 Rates and extent of poly(PEGDA10k-*co*-S1dsDNA) hydrogel swelling for different crosslink and hairpin concentrations. Hydrogels were polymerized with DNA crosslinks at 1.154, 0.750, or 0.250 mM (sequence set 1) and were incubated with hairpins at a final concentration of (a) 20 μM or (b) 60 μM H1 and H2 (sequence set 1, H1_S1_6/3 and H2_S1_6/3). Curves are the relative change in hydrogel side length after the addition of hairpins. Solid lines are the average of 3–6 hydrogels; dashed lines show 95% confidence intervals as determined by standard deviations.

rate and total extent of swelling were roughly proportional to the crosslink concentration in these experiments: The initial rates of swelling in the first 2 hours were 0.029 ± 0.001, 0.065 ± 0.005, and 0.087 ± 0.001 (standard error), and total extents of swelling were 0.24 ± 0.07, 0.58 ± 0.17, and 0.74 ± 0.09 (95% confidence interval) for 0.250, 0.750 and 1.154 mM of crosslinks respectively.

While faster swelling occurred in the first 10 hours, the final extent of hydrogel swelling was roughly the same when 60 μM of hairpins were added to the solution *vs.* when 20 μM of hairpins were added to the solution. This observation is consistent with the idea that the hairpins were in excess, and the total number of crosslinks limited the extent of swelling.

Self-limiting polymerization at crosslinks with terminator hairpins reduces swelling extent but can increase swelling speed

DNA hairpins facilitate continuous swelling in poly(Am-*co*-DNA) gels.[22] Swelling can also reach a specific extent at steady state through the use of terminator hairpins that can polymerize at crosslinks, much as the polymerizing hairpins do.[22,23] These terminator hairpins have non-complementary loop domains that, once inserted, prevent the insertion of subsequent hairpins (Text S2, ESI†) such that the final extent of uniaxial swelling is roughly inversely proportional to the fraction of terminator hairpins or the expected final average length of the crosslink in polymer units.[22] In PEG gels, crosslink extension may be limited even in the absence of these terminators by covalent linkages in the network that make crosslink extension unfavourable beyond some distance. We next asked how the presence of terminator hairpins affected the rate and extent of DNA-directed hydrogel swelling. We characterized poly(PEGDA10k-*co*-S1dsDNA1.154) hydrogel swelling induced by DNA hairpins with 2 or 10% mole fraction of terminator hairpins (H1_S1_ter and H2_S1_ter) and compared it to the rate of hydrogel swelling in the presence of the same concentration of hairpins without any terminators (Fig. 3). When both 2% and 10% terminators were added, the extent of swelling was reduced with respect to the extent of swelling in hairpin solution without terminators. The overall change in the trend of swelling extent on the inclusion of terminators is consistent with that observed elsewhere.[22] For the reported poly(Am-*co*-DNA) gels, the change in side length over original side length ($\Delta L/L_0$) for 0, 2, and 10% terminator are 3.8, 2.6, and 0.8, while the values for PEG gel are 0.82, 0.72, and 0.34.

Interestingly, we also observed a slight increase in the initial rate of swelling when terminators were used. This initial increase in rate could be due to terminating the extension of crosslinks at the hydrogel surface requires that hairpins incorporate into crosslinks deeper within the hydrogel, perhaps ensuring more even and thus faster swelling when measured by the size of the material as a whole. The inclusion of terminators can also reduce unwanted hairpin nucleation in the solution, reducing the size of DNA complexes and facilitating diffusion. Generally, terminators could be used to control the size of a PEG-*co*-DNA gel after DNA-directed swelling, and
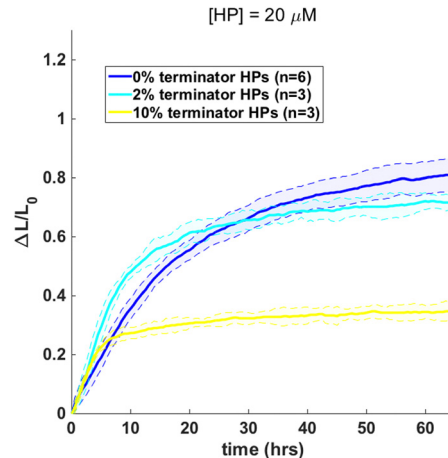

Fig. 3 Terminator hairpins can reduce the extent of hydrogel swelling with more terminators causing further reduction. The relative change in side length of poly(PEGDA10k-*co*-S1dsDNA1.154) hydrogel triangles incubated with set 1 hairpins (H1_S1_6/3 and H2_S1_6/3, H1_S1_ter and H2_S1_ter) at a total of 20 μM per hairpin type. Solid curves are each the averages of measurements of 3–6 hydrogels. Dashed lines show 95% confidence intervals as determined by standard deviations.

our results are consistent with the ability to achieve a wide range of final sizes by adjusting the terminator proportion.[22]

Effect of toehold length on hydrogel swelling

We next asked how the reaction rate and energy balance between the hairpins and crosslinks might be used to control the rate and extent of DNA-directed swelling, as varying the lengths of the toeholds on the hairpins and crosslinks would alter the kinetics and thermodynamics of the extension reaction.[27,28] Because hairpins and crosslinks react *via* a toehold-mediated 4-way branch migration, there are 2 toeholds (termed primary, Fig. 4a, and secondary, Fig. 5a) involved in the process that could each affect insertion reaction kinetics and thermodynamics. In our initial experiments, the primary toehold was 6 nucleotides long and the secondary toehold was 3 nucleotides long.

We began by investigating how lengthening the primary toehold would change the speed and extent of DNA-directed swelling. We designed a set of DNA crosslinks, termed sequence set 2, with 10 nucleotide-long primary initiating toeholds on one of the crosslink strands that could hybridize primary toeholds of up to these lengths (A_S2 and R_S2, set 2_v1, Table S1, ESI†).

The hydrogels containing set 2 crosslinks swelled at a slightly slower rate, and a lower final degree in response to the same concentration of hairpins with the same toehold lengths compared to set 1 (Fig. S12, ESI†). Hydrogels containing set 2 crosslinks achieved an average relative change in hydrogel side length of about 0.65 in 60 hours compared to a relative change of 0.8 for hydrogels containing sequence set 1 crosslinks. This difference is potentially due to differences in the two sets' sequences: there is only one G/C pair in both primary and secondary initiating toeholds of the set 2 sequence, while the
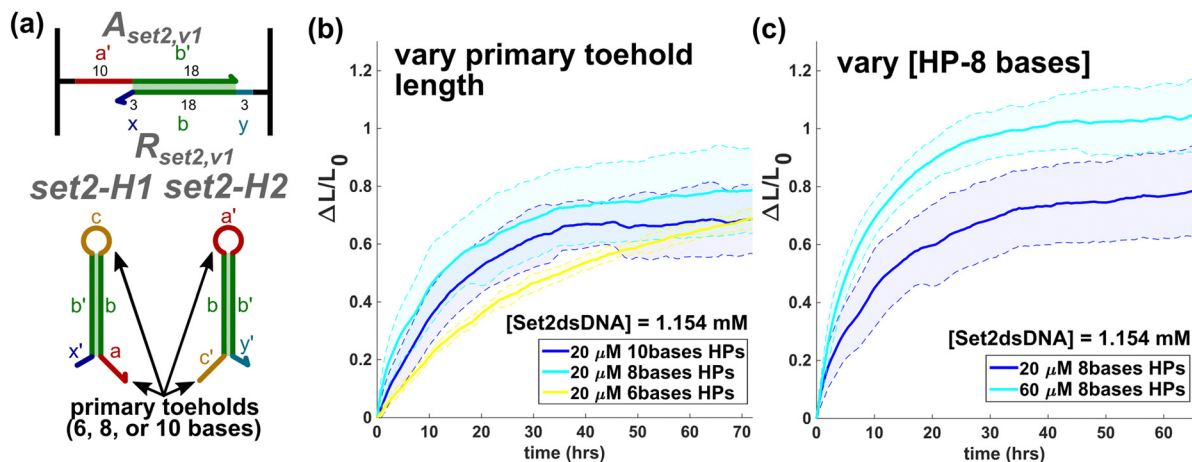
Fig. 4 The length of the hairpin's primary toehold affects the degree and rate of hydrogel swelling. (a) Poly(PEGDA10k-*co*-S2_v1dsDNA1.154) hydrogels were polymerized with crosslinks containing 10 bases primary toeholds and 3 bases secondary toeholds. Numbers indicate the number of bases in those domains in the crosslink. Hairpins were designed to have 6, 8, or 10 base-long primary toeholds that can react with the crosslinks for hairpin insertion. (b) Relative change in hydrogel side length for hydrogels polymerized with 1.154 mM of the crosslinks in (a) incubated with 20 μM of hairpins containing 6 (H1_S2_6/3 and H2_S2_6/3), 8 (H1_S2_8/3 and H2_S2_8/3), or 10 (H1_S2_10/3 and H2_S2_10/3) base-long primary toeholds. (c) Relative change in hydrogel side length for hydrogels polymerized with 1.154 mM of the crosslinks in (a) incubated with 20 or 60 μM 8 bases primary toehold hairpins (H1_S2_8/3 and H2_S2_8/3). Solid lines are the average of 3–5 hydrogels; dashed lines show 95% confidence intervals as determined by standard deviations.

set 1 crosslink sequence contains three G/C pairs. Toeholds with a lower G/C content have a less negative ΔG of hybridization, leading to a slower rate of strand displacement.[28,36]

We used the set 2_v1 crosslinks to measure the difference in swelling rate and extent when hairpins with 6, 8, or 10 base primary initiating toeholds direct swelling (H1_S2_6/3 and H2_S2_6/3, H1_S2_8/3 and H2_S2_8/3, H1_S2_10/3 and H2_S2_10/3, Fig. 4a). Hydrogels incubated with hairpins with 8 and 10 base-long primary toeholds swelled slightly faster and to a greater extent by 70 hours than the hydrogels incubated with hairpins with 6 base primary toehold (Fig. 4b). These slight differences are consistent with studies of 4-way branch migration, in which slight increases (on the order of 2-fold) in reaction rate are observed when extending either the primary or secondary toehold length to more than 4–5 bases.[27,37] The relatively large size of the loop (10 bases) in the 10 base primary toehold hairpin could allow undesired hairpin-hairpin polymerization reactions,[36] leading to a slight decrease in the effective hairpin concentration and the overall rate of insertion into the crosslinks. Interestingly, unlike poly(PEGDA10k-*co*-S1dsDNA1.154) hydrogels incubated with 6 base-long primary toehold hairpins (Fig. 2), poly(PEGDA10k-*co*-S2_v1dsDNA1.154) hydrogels incubated with 8 base-long primary toehold hairpins had an extent of swelling that was dependent upon hairpin concentration (Fig. 4c). The rate and the extent of swelling of poly(PEGDA10k-*co*-S5dsDNA1.154) hydrogels increased when the concentration of 8 base-long primary toehold hairpins was increased from 20 μM per hairpin to 60 μM per hairpin. Poly(PEGDA10k-*co*-S2dsDNA1.154) hydrogels reached a uniaxial swelling ratio greater than 1 within 24 hours of incubation with 60 μM per hairpin.

Since increasing the length of the primary toehold from 6 to 8 bases led to a moderate increase in the rate and extent of

hydrogel swelling, we hypothesized that increasing the length of the secondary toehold on the hairpins and crosslinks could increase the rate and extent of swelling even further. To examine the effect of changing the length of the secondary toehold domain, we designed a variant of the sequence set 2 crosslink v2, which had a 6 base-long domain for binding a secondary toehold in addition to the 10- base long domain for binding a primary toehold (A_S2 and R_S2x6, Fig. 5a and Table S1, ESI†). We then measured the extent of swelling of poly(PEG-*co*-S2_v1dsDNA1.154) hydrogels when incubated with 20 μM hairpins with 6 base-long secondary toeholds and either 6 or 8 base-long primary toeholds (H1_S2_8/6 and H2_S2_8/6, H1_S2_6/6 and H2_S2_6/6, Fig. 5b). The length of the secondary toehold was not increased beyond 6 bases because equilibrium analysis using NUPACK showed an increase in hybridization between complementary secondary toeholds (*e.g.*, *x* to *x'* in Fig. 1a) longer than 6 bases at the insertion site that could inhibit hairpin insertion.

Increasing the lengths of the secondary toeholds from 3 to 6 bases resulted in a slight but not statistically significant increase in the degree of hydrogel swelling. No increase in the rate of swelling was observed. In contrast, in studies of 4-way branch migration with a 6 base-long primary toehold in which the secondary toehold length was increased from 4 to 6 bases, at least 4-fold increases in the reaction rate constant were observed and the ΔG° for the reaction decreased from either -8.63 or -10.23 kcal mol^{-1} to -12.45 kcal mol^{-1}.[27] These results and those in Fig. 4 suggest that the kinetics of branch migration in crosslinked hydrogels, where molecular crowding is a factor, may occur following different dependencies than those occurring in free solution. For example, recent studies have shown that the ΔG° of DNA hybridization decrease by up to 50% in 40 wt% PEG solutions.[38] There is, however,
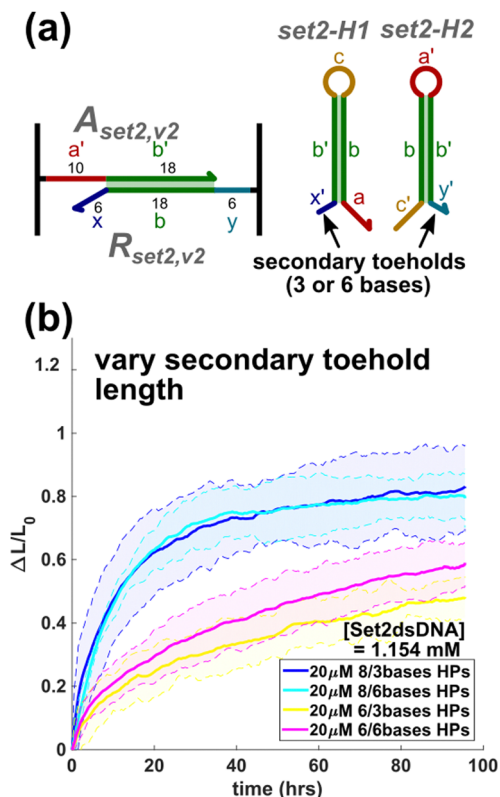
Fig. 5 Effect of the length of the hairpin's secondary toehold on the degree and rate of hydrogel swelling. (a) Poly(PEGDA10k-*co*-S2_v2ds DNA1.154) hydrogels were swollen by hairpins with different primary and secondary toehold lengths. (b) Relative change in hydrogel side lengths of hydrogels polymerized with 1.154 mM crosslinks in (a) incubated with 20 μM of hairpins with different primary/secondary toehold lengths (in bases). Solid lines are the average extents of swelling of 3 hydrogels; dashed lines show 95% confidence intervals as determined by standard deviations.
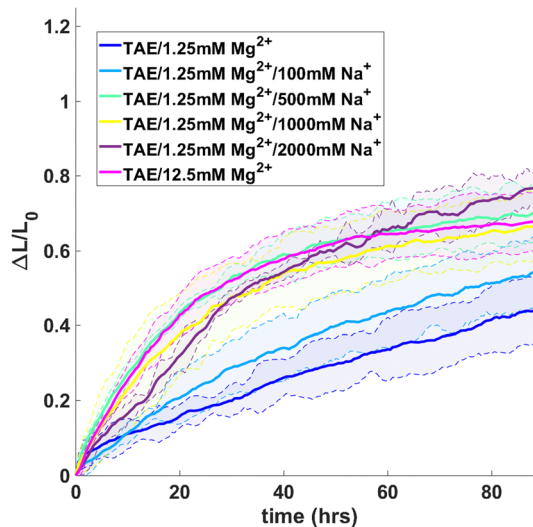

Fig. 6 Swelling kinetics for poly(PEGDA10k-*co*-S2dsDNA1.154) hydrogel using 20 μM of H1_S2_6/3 and H2_S2_6/3 in: 1x TAE/1.25 mM Mg^{2+}, 1x TAE/1.25 mM Mg^{2+}/100 mM Na$^+$, 1x TAE/1.25 mM Mg^{2+}/500 mM Na$^+$, 1x TAE/1.25 mM Mg^{2+}/1000 mM Na$^+$, 1x TAE/1.25 mM Mg^{2+}/2000 mM Na$^+$, and 1x TAE/12.5 mM Mg^{2+} (TAEM). Solid curves are the averages of measurements of 3 hydrogels each. Dashed lines show 95% confidence intervals as determined by standard deviations.

little data about kinetic parameters, and the rates of 4-way toehold branch migration even in solution are much less well-studied than other toehold-mediated branch migration processes. More studies would likely be needed on both types of processes to establish a systematic comparison.

Salt concentration

Like toehold length, the concentration and type of cations in solution are also strong determinants of the kinetics and thermodynamics of DNA strand displacement and toehold-mediated branch migration.[28,30] A commonly accepted model is that the $\Delta G°$ of DNA hybridization increases with the log of cation (either Na$^+$ or Mg^{2+}) concentration and that these increases occur per pair of bases and are well-modeled by the nearest neighbor model of DNA hybridization.[39,40] The salt concentration would therefore be expected to change the extent of swelling and possibly the rate of swelling.

We tested the swelling of poly(PEGDA10k-*co*-S2dsDNA1.154) hydrogels in several different buffers with different sodium concentrations (1x TAE/1.25mM Mg^{2+}, 1x TAE/1.25 mM Mg^{2+}/ 100 mM Na$^+$, 1x TAE/1.25 mM Mg^{2+}/500 mM Na$^+$, 1x TAE/1.25 mM

Mg^{2+}/1000 mM Na$^+$, 1x TAE/1.25 mM Mg^{2+}/2000 mM Na$^+$). To control for the effects of ion-dependent swelling of the PEG matrix, we initially incubated each hydrogel in each buffer for at least 24 hours before adding 20 μM of each DNA hairpins (H1_S2_6/3 and H2_S2_6/3). The results in Fig. 6 indicate that the initial rate and the extent of swelling after 80 hours increased with increasing Na$^+$ concentration in the range of 0 mM to 500 mM. Furthermore, the swelling behaviour of gels in 2M Na$^+$ was similar to that of 500 mM Na$^+$, 1 M Na$^+$, and the control group using 1x TAE/12.5 mM Mg^{2+} (TAEM). We also tested the swelling of poly(PEGDA10k-*co*-S1dsDNA1.154) hydrogels in several different buffers: 1x TAEM, 1xTAE/100mM Na$^+$, 1x sodium phosphate–sodium chloride (SPSC, 1 M NaCl, 50 mM Na$_2$HPO$_4$; pH 7.5) and PBS (Fig. S1, ESI†). No significant further swelling due to the ions alone was observed. Our measurements indicate that the hydrogels incubated with 1x TAEM and 1x SPSC exhibit similar rates and extents of swelling, while the rates and extents of swelling of hydrogels in TAE/Na$^+$ and PBS were significantly lower. SPSC has a higher concentration of sodium (1 M) than either PBS (137 mM) or TAE/ 100 mM Na$^+$, so would be expected to allow stronger DNA hybridization as would TAE/12.5 mM Mg^{2+} given the comparative effects of Mg^{2+} and Na$^+$ ions on hybridization thermodynamics.[41] We further tested whether varying the concentration of Mg^{2+} in TAE buffer would affect the swelling, and the result in Fig. S2 (ESI†) shows that the rate and degree of swelling were almost the same. This finding suggests that the amount of Mg^{2+} can be adjusted as needed, for example, to deploy DNA polymerization gels in environments such as cell culture without significantly altering this type of DNA strand displacement process.

Expanding hydrogels containing ssDNA anchors

Finally, we asked whether hydrogel swelling could be induced by DNA hairpins with specific sequences when the hairpin polymerization initiators within the hydrogel were not cross-links but single strands of DNA (Fig. 7a). A single-stranded DNA (ssDNA) initiator initiates the polymerization of the hairpins only through the primary toehold. Hairpin insertion then proceeds through a 3-way branch migration pathway rather than *via* 4-way branch migration reactions as in the case of the double-stranded initiator.

When polymerizing hairpins are added to hydrogels containing single-stranded DNA initiators, the hairpins should incorporate into the hydrogel as in the case with double-stranded initiator complexes. But when single-stranded initiators are used, this incorporation does not extend hydrogel crosslinks. Because the ssDNA is not anchored to the gel on both ends, it can only indirectly, through mixing energy and excluded volume, alter the shape of the hydrogel network. If these effects are relatively unimportant for hydrogel swelling, less or even no hydrogel swelling might be expected to occur during such a process.

We prepared PEGDA10k hydrogels polymerized with either 0.750, 1.154, or 2.308 mM of the sequence set 2 A strand (A_S2, Table S1, ESI†), which contains the primary toehold (Fig. 7a). We incubated these poly(PEGDA10k-*co*-S2ssDNA) hydrogels (sequence set 2 v1, Table S1, ESI†) with 20 µM 8 base primary toehold hairpins (H1_S2_8/3 and H2_S2_8/3). The hydrogels did swell, although to a lesser extent than when double-stranded crosslinks were used. The extent of hydrogel swelling

was dependent upon the concentration of ssDNA initiators polymerized into the hydrogel (Fig. 7b). When the hydrogels were incubated with 10 base primary toehold hairpins (H1_S2_10/3 and H2_S2_10/3), the relative changes in side length of poly(PEGDA10k-*co*-S2ssDNA) hydrogels were about 0.3 to 0.6 after 60 hours when the initiator concentrations were 1.154 mM to 2.308 mM respectively (Fig. 7c). The roughly linear dependence of swelling extent on crosslink concentration in each experiment is consistent with the linear dependence on swelling extent from double-stranded crosslinks.

While the extent of swelling from double-stranded crosslinks was not highly dependent on toehold length, poly(PEGDA10k-*co*-S2ssDNA1.154) hydrogels incubated with hairpins with 10 base primary toeholds (H1_S2_10/3 and H2_S2_10/3) swelled significantly more than those incubated with hairpins with 6 base (H1_S2_6/3 and H2_S2_6/3) or 8 base (H1_S2_8/3 and H2_S2_8/3) primary toeholds (Fig. 7d). Hydrogels swelled faster, however, when incubated with hairpins with 6 base primary toeholds. In the free solution, the rate of a 3-way branch migration reaction initiated by 6 base toeholds is approximately 2 fold lower than those of 8 and 10 base toeholds (whose rates are indistinguishable).[28] The relative similarity of the rates of swelling in response to the hairpins with the 6 and 8 base toeholds is consistent with the rates of strand displacement reactions, but the slower rate of swelling of hydrogels in response to the hairpins with 10 base toeholds is not.

The hydrogels polymerized with the ssDNA initiators also showed a slight dependence upon the concentration of polymerizing hairpins added to the hydrogel. The change in side
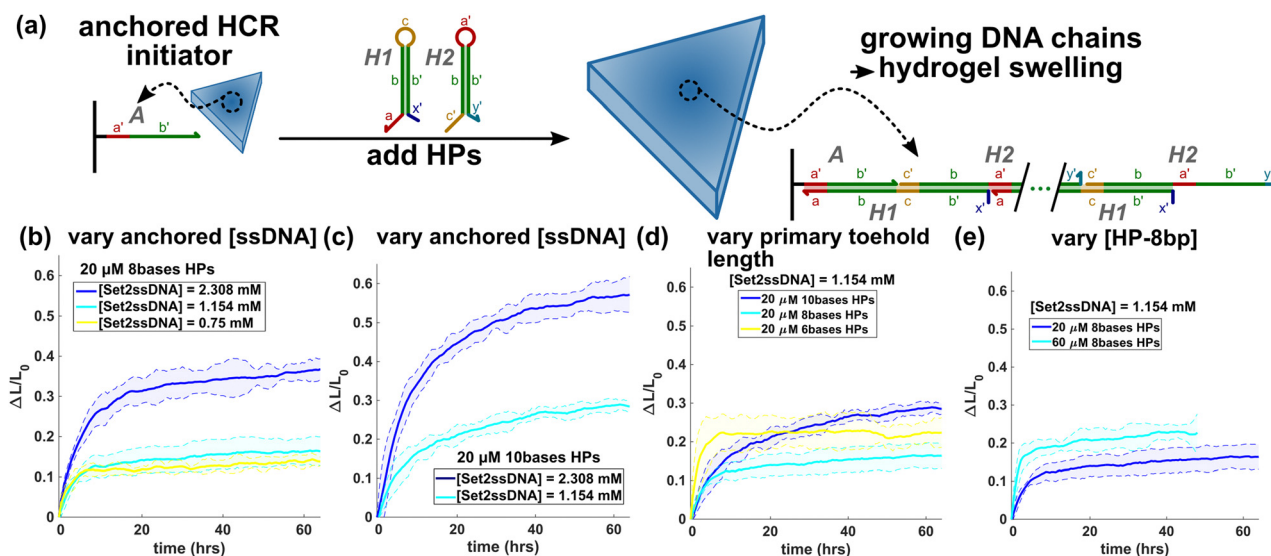

Fig. 7 DNA-directed swelling of hydrogels with anchored single-stranded HCR initiators. (a) Single-stranded DNA that is capable of initiating an HCR process is anchored to hydrogels during hydrogel polymerization. Hairpins sequentially bind to these anchored DNA strands *via* a hybridization chain reaction, inducing hydrogel swelling. (b) Relative change in the side length of poly(PEGDA10k-*co*-S2ssA^{ac}) hydrogel hydrogels with varying concentrations of anchored ssDNA HCR initiators. Hydrogels were incubated with 20 µM of 8 base-long primary toehold hairpins. (c) Relative change in side length of poly(PEGDA10k-*co*-S2ssA^{ac}) hydrogels with varying concentrations of anchored ssDNA HCR initiators incubated with 20 µM of 10 base-long primary toehold hairpins. (d) Relative change in side length of poly(PEGDA10k-*co*-S2ssA^{ac}1.154) hydrogels incubated with 20 µM hairpins with 6, 8, or 10 base-long primary toeholds. (e) Relative change in side length of poly(PEGDA10k-*co*-S2ssA^{ac}1.154) hydrogels incubated with 20 or 60 µM 8 base primary toehold hairpins. Solid lines are the average of 3–5 hydrogels; dashed lines show 95% confidence intervals as determined by standard deviations.

length of poly(PEGDA10k-*co*-S2ssDNA1.154) hydrogels increased from 0.15 to 0.23 in 50 hours when the concentration of 8 base primary toehold hairpins was increased from 20 μM to 60 μM per hairpin (Fig. 7e). This change in the extent of swelling is consistent with the difference in the extent of swelling observed when the concentrations of the same hairpins were varied with double-stranded crosslinks (Fig. 4c).

We used gel electrophoresis to compare the extent of polymerization resulting from this reaction in free solution when the initiator is single-stranded *vs.* when it is double-stranded (Fig. S13, ESI†). The extent of polymerization is less when using a single-stranded initiator than when using a double-stranded initiator. Further, in both cases, there is an abundance of leftover hairpin monomer. This observation could be explained by the fact that the polymerization step from a single-stranded initiator is less forward-driven than the polymerization step from a double-stranded initiator is, due to the absence of the toeholds *x* and *y* in the R strand when a single-stranded initiator is used.

Dependence of design rules on hydrogel form

To explore whether the size of the hydrogel affects the degree of swelling, we fabricated hydrogel triangles with the same side length (1 mm) and varying thicknesses of 60 μm, 160 μm (as used in other gels in this paper for comparison), and 320 μm. The final swelling characteristics showed that the 60 μm-thick gels had swelled approximately 30% more after 40 hours as compared to the 160 μm-thick gels, while the 320 μm-thick gels had swelled considerably less (Fig. S3, ESI†). This reduction of swelling with increasing gel thickness could be due to a higher crosslinking density in the thicker gels as considerably more energy was used to polymerize the 320 μm-thick gels during photopatterning than was used to pattern the thinner gels (1200 mJ cm^{-2} *vs.* 600 mJ cm^{-2}). The lesser extent of swelling in thicker gels could also be due to the hairpins not penetrating all the way into the 320 μm-thick gels, causing more swelling on the borders of the gels, preventing overall area expansion. Such inhomogeneous swelling was observed with Am-DNA gel particles with a 1 mm diameter during swelling (Video S1 and S2, ESI†). Both videos show that the swelling happens successively from outside to inside of a material, while increasing the terminator percentage from 2% to 10% increases the rate of swelling in the interior part of the gel particle. The initial rates of swelling of the 60 μm- and 160 μm- thick gels were largely the same, which indicates that diffusion is not a major speed-limiting factor during the swelling process of thinner gels. However, the swelling process of a thicker (320 μm) gel could be diffusion-limited as the Damköhler number (Da) is a quadratic function of thickness.[42] The Damköhler number (Da) is a dimensionless quantity used in chemical engineering and fluid mechanics to characterize the relative rates of reaction and diffusion or transport processes in a system. As Da = (Diffusion time)/(Reaction time), high Da values indicate a diffusion-limited distribution.

To examine the extent to which the swelling process depends on the shape of the hydrogel, we repeated some comparisons using millimeter-scale hydrogel particles. PEGDA10k hydrogel particles with diameters of about a millimeter containing different concentrations of S1dsDNA crosslinks were prepared using a previously developed droplet-based synthesis method.[24] As expected, the DNA-directed swelling of the hydrogel particles was slower than the swelling of the smaller triangles, making the final extent of swelling of the particles challenging to discern in some cases. The measured extents of swelling of the particles were also somewhat more varied than the measured extents of swelling of the triangles. This variation may be due to the fact that the particles have slight aberrations from a spherical shape so that rotations of the particles change their apparent sizes. As photopatterned gels offer improved consistency and reproducibility in conducting gel swelling assessments, we suggest employing this fabrication technique in future gel swelling characterization studies.

Nonetheless, the measurements of the extent of swelling of the particles showed trends with respect to DNA concentration and crosslink form that were qualitatively similar to those observed in experiments with hydrogel triangles while having larger sample variance (Fig. S4 and S5, ESI†). Salinity also affected the swelling kinetics for gel particles, where hairpins in 1x TAEM and 1x SPSC buffer performed similarly while better than TAE/Na$^+$ and PBS (Fig. S6 and S7, ESI†).

We also prepared poly(PEGDA10k-*co*-S1dsA^{ac}R$^{no\text{-}ac}$1.154) hydrogel particles where the AR complex (see Fig. 1a) was covalently linked to the PEGDA matrix only through the A strand. Interestingly, the particles swelled at essentially the same rate and extent as poly(PEGDA10k-*co*-S1dsDNA1.154) particles when incubated with 20 μM hairpins (Fig. S8b, ESI†). This observation leads to an intriguing hypothesis about whether the R connection is necessary for swelling. In another experiment depicted in Fig. S9, we compared the swelling of different poly(Am-*co*-DNA) hydrogels in response to S1 hairpins. All hydrogels contained dsDNA that is conjugated to the polymer backbone on both strands. The first type of hydrogel had S1 dsDNA conjugated by both ends whereas the second group had S3 dsDNA conjugated by both ends. The second group of hydrogels also had S1 dsDNA that is only conjugated to the polymer backbone by the A strand side. When adding S1 hairpins to these two groups of hydrogels, we would therefore expect that both would swell and that the first group would swell by extension of mechanically coupled crosslinks, whereas the second would swell *via* polymer extension through a simple polyelectrolyte effect. If the R connection is unimportant, we would expect the swelling to be the same. However, Fig. S9 (ESI†) shows the gels with the doubly conjugated S1 dsDNA exhibit 4 times greater swelling on average than the gels with the dsDNA conjugated only to the A strand. It, therefore, appears we need to better understand the role of the R connection and R strand in different materials and contexts. Another potential factor in the R connection is that DNA crosslinks in a gel contribute to the mechanical modulus. We previously demonstrated that the presence of dsDNA crosslinks can significantly increase a hydrogel's modulus by 20–45%.[23] If the gel's swelling was entirely a polyelectrolyte effect, then the

use of the dsDNA as a crosslink should actually slightly inhibit swelling as compared to the inclusion of the dsDNA conjugated by only one strand.

Conclusions and outlook

Here, we have investigated how altering features of the DNA crosslinks and hairpins that control the kinetics and degree of DNA strand displacement, in turn, affect the rate and extent of DNA polymerization gel swelling.

While none of the parameters we tested induced order-of-magnitude or greater changes in the rate of hydrogel swelling, we identified design parameters with the strongest effects on swelling rates and extent. Of note, the concentration of anchored DNA inside hydrogels had the greatest effect on both the kinetics and degree of swelling. Next in importance was the concentration of hairpin fuel used to swell the gels and its effect was dependent on the sequences. The lengths of the toeholds were found to be the least important factor.

We also showed that hydrogels could be expanded using the HCR process by integrating single-stranded DNA initiators or single side-anchored dsDNA into the hydrogel matrix. Using these types of initiators decreases the number of acrydite-modified DNA strands by half, reducing the DNA-responsive hydrogel device production cost.

When we varied the gel thicknesses (60 μm or 160 μm), we discovered that these factors have limited effects on the swelling process. Larger gel dimensions (*e.g.*, a 320 μm thickness) or different gel shapes (sheets or sphere-like particles) that limit diffusion can largely decrease the gel swelling.

Finally, it is the DNA polymerization at DNA crosslinks that drives the swelling of DNA polymerization gels. The swelling process involves the transport of water and ions and the reorganization of the polymer network. In future studies, the ability to choose the physical properties of the gel (*e.g.*, diffusion coefficients of different molecules, locking stretch–the value of the chain stretch when it achieves maximum extended length, and Flory–Huggins interaction parameter) could help us develop a strategy that considers the combined thermodynamic and kinetic effects to allow for the rational design of such responsive polymerization gels.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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